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Award Number: W81XWH-04-1-0474

TITLE: Functional Geno,ic Analysis of Breast Cancer Cell Tumorigenicity Using a Noval Gene Silencing Resource

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REPORT DATE: April 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2006	Annual	31 Mar 05 – 30 Mar 06

4. TITLE AND SUBTITLE
Functional Geno,ic Analysis of Breast Cancer Cell Tumorigenicity Using a Noval Gene Silencing Resource

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0474

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Research Foundation
State University of New York
Albany NY 12201-0009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The year 1 aims were primarily concerned with collecting shRNA constructs so that a small number that have inhibitory effects on breast cancer progression in vivo models could be identified. We have collated a set of encoded hairpins targeting genes overexpressed in ErbB-2 positive breast tumors. We have also spent considerable effort testing the compatibility of several assays for cellular correlates of tumorigenicty with high throughput gene transfer. To date, we have retrieved 65 shRNA constructs targeting 51 of the genes overexpressed ErbB2-positive breast cancer cells and tested them for effects on cell proliferation in a screen in BT474 cells. Year 2 of funding resulted in tests of the specificity of the shRNAs' negative impact on the growth of Erb-B2 positive breast cancer cells. Effects of these shRNAs on normal cells and other breast cancer cell lines, identified approximately 20 shRNAs that specifically inhibit Erb-B2 positive breast cancer cells target genes. Several of these gene targets are known to be important to a variety of cancers. Two novel genes are interesting because they give new insight into a pathway that can be exploited as a potential therapeutic target.

15. SUBJECT TERMS
FUNCTIONAL GENOMICS, CELL SIGNALING, GENE THERAPY, RNAI

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
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Introduction

The recent explosion of genome sequences and bioinformatic data has changed the nature of mammalian genetics. Systematic investigation of expression patterns of thousands of genes in breast tumors using cDNA microarrays and serial analysis of gene expression has yielded a long list of genes whose increased activity is correlated with the occurrence of breast cancer (1-4). In notable cases, associations between gene expression levels and clinical outcome have been determined. In most cases, however, the information is only correlative and serves as a rough guide to genes that might positively influence some aspect of breast cancer.

We are working towards identifying which of the nearly 100 genes typically overexpressed in ErbB-2 positive breast tumors are important for the survival and aggressiveness of breast cancer cells. Our approach makes use of DNA-encoded short hairpin RNAs that elicit an RNA interference silencing of the overexpressed genes. Using high throughput methodologies, we have constructed a library of sequence-verified hairpins that target most human genes (5). Constructs from the library have been used for the systematic validation of breast cancer molecular profiling data in ErbB-2 positive BT 474 cells. The overall objective of this proposal is to identify hairpins that have inhibitory effects on breast cancer progression in *in vivo* models as this would add to our understanding of the genes involved in breast cancer and identify their products as potential small molecule therapeutic targets. It would also pave the way for testing of the hairpins that we identify as gene therapeutics.

The underlying hypothesis of this proposal is that functional genomic screening with DNA-encoded RNA interference can identify those genes that breast cancer cells actually rely on to cause cancer. We will systematically target the most commonly overexpressed genes in breast cancer cells especially those that are overexpressed in association with ErbB-2 amplification. Many of these genes are likely to be involved in granting some sort of survival advantage to these cells during cancer progression. Silencing key genes in this group should reverse the cancer phenotype. In this way, we expect to gain insights into which genes are actually required by breast cancer cells to cause cancer and would therefore make excellent therapeutic targets. Moreover, it may contribute directly to the development of gene-based therapeutics by identifying short hairpin RNAs that could someday be used clinically.

Body

The year 1 aims were primarily concerned with collecting shRNA constructs so that a small number that have inhibitory effects on breast cancer progression *in vivo* models could be identified. The major goal of this time period as set forth in the Statement of Work was: **Creation of a set of encoded hairpins targeting genes overexpressed in ErbB-2 positive breast tumors.** Months 1-12 of the proposal were to be spent in the creation of a set of encoded hairpins that targeted those genes commonly overexpressed in ErbB-2 positive breast tumors. We had planned to make use of a library of over 20,000 sequence-verified hairpins that we had constructed. This library targeted approximately 10,000 human genes. Included were those genes which encoded all of the protein classes that are viewed to be accessible to medicinal chemistry. More importantly, the majority of sequences identified in ErbB-2 positive breast cancer molecular profiling experiments were also represented in the library.

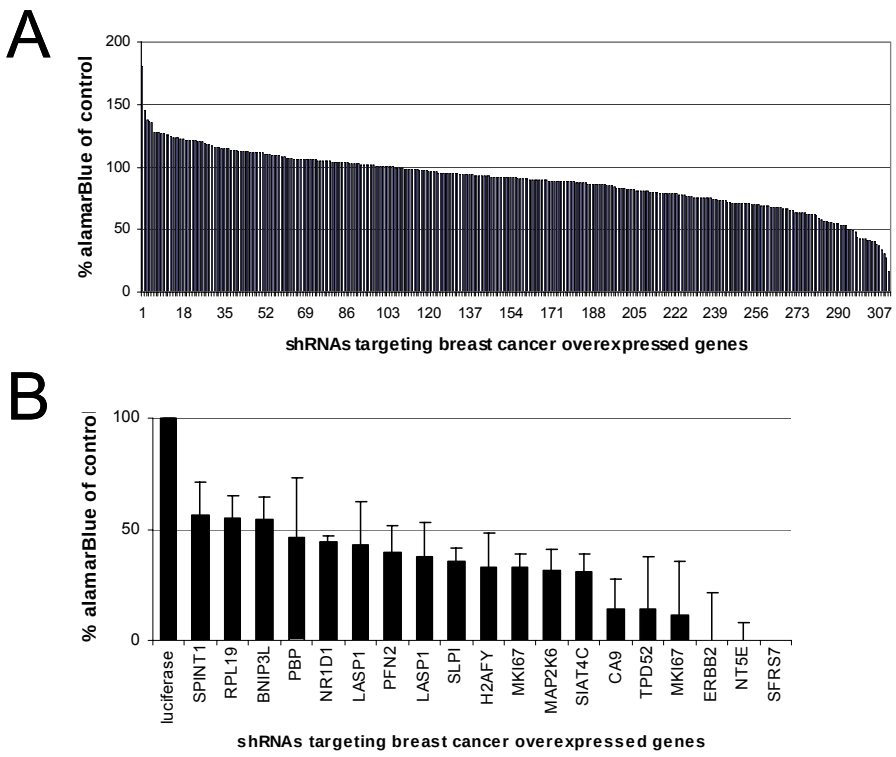

Figure 1. The RNAi screen for breast cancer overexpressed genes. (A) ERBB2-positive BT474 breast cancer cells were seeded in 96-well plates and were transfected by FuGENE6 with a total of 323 shRNA constructs targeting 144 genes known to be overexpressed in breast cancer. Non-specific shRNA constructs targeting the firefly luciferase gene were used as negative controls and shRNAs targeting the well-established oncogene ERBB2 were used as positive controls. All hairpins were co-transfected with a MSCV construct expressing green fluorescent protein (GFP) in order to monitor for transfection efficiency. After four days cells were assayed for proliferation by using alamarBlue (Biosource). Results were normalized for transfection efficiency and were represented as percentage of the luciferase control. All transfections were performed in triplicate and in a triplicate of wells for each experiment and the averages and standard deviations were calculated. Results shown here are sorted on the basis of the effect. (B) Results were confirmed in a second round of transfections on BT474 cells for most of the shRNAs that produced significant alamarBlue decrease in the initial screen. Experiments were performed as above and results are represented also as described above.

Although the original plan was to collect these from an existing short hairpin RNA library and synthesize any missing constructs for genes, advances in RNAi technology simplified this process. A second generation library was constructed in a vector that produces more infective viral particles and expresses short hairpin RNAs with better silencing properties. This library is available through a commercial vendor. As a developer of the original shRNA library detailed in the proposal, I have received the entire library targeting virtually every gene in the human library at cost from the supplier. Although this resource became available early this year, it has simplified assembling a sublibrary of shRNAs targeting overexpressed genes.

In the first year, we also spent considerable effort testing the compatibility of several assays for cellular correlates of tumorigenicty with high throughput gene transfer. Among these are standard soft agar assays, DAPI staining to quantify apoptotic nuclei, Cyquant assays, Rb and E2F luciferase-based reporter cell cycle progression assays, Alamar blue proliferation stains and live cell counts. Although the assays are straightforward, most were insufficiently robust for large scale analysis. Instead we have found that using Alamar blue (Biosource) reduction that is normalized to GFP transfection efficiency to measure shRNA containing cell proliferation is the most workable assay in high throughput. This assay is by far the most robust.

RNAi screen for breast cancer overexpressed genes

An RNAi approach was implemented for a functional screen of 144 genes previously reported to be up-regulated in ERBB2-positive breast cancer. A total of 323 short-hairpin RNAs (shRNAs) built in the pSHAG-MAGIC 2 (pSM2) vector were used to target these genes in the ERBB2-positive, BT474 breast cancer cell line. Hairpins targeting the firefly luciferase gene were used as non-specific negative controls, while hairpins targeting the ERBB2 gene were used as positive controls, since it has been shown that RNAi against

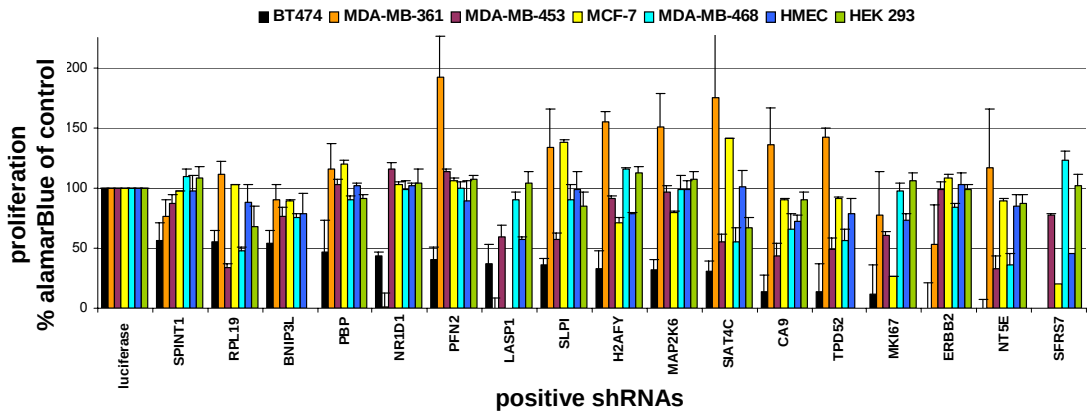

Figure 2. The shRNAs with confirmed impact on BT474 cells were used to transfect four other breast cancer cell lines (MDA-MB-361; MDA-MB-453; MCF-7; MDA-MB-468), human mammary epithelial cells (HMEC) and a non-breast cell line (HEK 293). Experimental approach was the same as above. In most cases, shRNAs significantly had an effect on breast cancer cells and not on primary or non-breast cells; in addition, the ERBB2 shRNA had a significant effect only in the ErbB2-overexpressing cell lines (BT474 and MDA-MB-361). Interestingly, two other hairpins targeting PBP (PPAR binding protein) and NR1D1 (nuclear receptor subfamily 1, group D, member 1), which are known to both control the adipogenesis pathway, specifically decreased alamarBlue of the ERBB2-positive cells, as well.

ERBB2 significantly inhibits proliferation and induces apoptosis in BT474 cells (Faltus *et al.*, 2004).

shRNA-transfected cells were monitored for changes in the proliferation marker alamarBlue after four days of transfection, since this cell line exhibits a population doubling time of ~100 hours. Results showed that most of the hairpins resulted in decrease in alamarBlue numbers comparing to the luciferase control, with a small number of them resulting in a difference of more than 50% (Fig. 1A). The hairpins that produced the most significant effect were used in a second round of transfections in low-throughput scale that confirmed the results from the initial screen, in most cases (Fig. 1B). One of the hairpins with significant impact on BT474 cells was a construct targeting ERBB2, as was expected. In this group of hairpins were also constructs targeting genes with established significance to the cancer cell phenotype, such as the tumor protein D52 (TPD52), the antigen identified by monoclonal antibody Ki-67 (MKI67), and the carbonic anhydrase IX (CA9). Interestingly, similar effects were produced by a number of hairpins targeting genes with no known direct relationship to cancer, like the PPAR-binding protein (PBP), the nuclear receptor subfamily 1, group D, member 1 (NR1D1 or Rev-Erbα), the serine peptidase inhibitor, Kunitz type 1 (SPINT1), and the splicing factor, arginine/serine-rich 7 (SFRS7) (Fig. 1B). These results validated the approach.

We further sought to examine the effect of the hairpins that produced the best results from the screen on a number of breast cancer cell lines , of human mammary epithelial cells and of a non-breast tumorigenic cell line, HEK 293FT cells (Fig. 1C). Hairpins targeting TPD52, MKI67 or CA9 seem to have a broad effect on most cell types. However, the ERBB2 hairpin specifically affected ERBB2-positive cells (BT474 and MDA-MB-361). Similarly, hairpins targeting PBP and NR1D1 had an impact specifically on BT474 cells, whereas NR1D1 dramatically affected MDA-MB-361 proliferation, as well. These two genes have been shown to be functionally related, since they are both implicated in adipocyte differentiation; PBP is a co-activator of the PPAR family of transcriptional factors, mainly of PPARγ (Zhu *et al.*, 1997) that positively regulates expression of adipogenic genes, such as NR1D1 (Fontaine *et al.*, 2003). The latter usually acts as a negative regulator of transcription, inhibiting expression of anti-adipogenic genes (Laitinen *et al.*, 2005; Chawla & Lazar, 1993). NR1D1 has been also shown to be a major component of the circadian clock (Yin & Lazar, 2005; Yin *et al.*, 2006; Ueda *et al.*, 2005). Recently, it has been shown that some components of the circadian clock are play important roles in the genesis of cancer cell phenotypes (Gery *et al.*, 2006; Fu *et al.*, 2002; Gery *et al.*, 2005). We expect that further study of the PBP NR1D1 pathway will add to our understanding of breast cancer and identify potential small molecule therapeutic targets.

PBP and NR1D1 hairpins cause decreased viability of BT474 cells

In order to further clarify cell phenotype after PBP and NR1D1 hairpin treatments, we employed additional approaches to study their effects on BT474 cells. Fluorescent microscopy after four days of hairpin and MSCV-GFP co-transfection showed that the number of transfected cells was significantly reduced when compared to the luciferase hairpin control. This was most noticeable when the NR1D1 hairpin was used (Fig. 2A). FACS analysis of cells transfected as above also showed that the number of GFP-

positive cells was significantly lower when the PBP and NR1D1 hairpins were used, when compared to the luciferase hairpin control (Fig. 2B). FACS analysis also showed an increased percentage of apoptotic (sub-G1) cells (Fig. 2B). In addition, luciferase assay on cells co-transfected with constructs expressing firefly and renilla luciferase genes showed a significant decrease of total luciferase activity when the NR1D1 hairpin was used, when compared to empty vector control (pSM2e) (Fig. 2C). The above indicate that hairpins that down-regulate PBP and NR1D1 genes (Fig. 2D) result in decreased viability and increased apoptosis of the BT474 breast cancer cells.

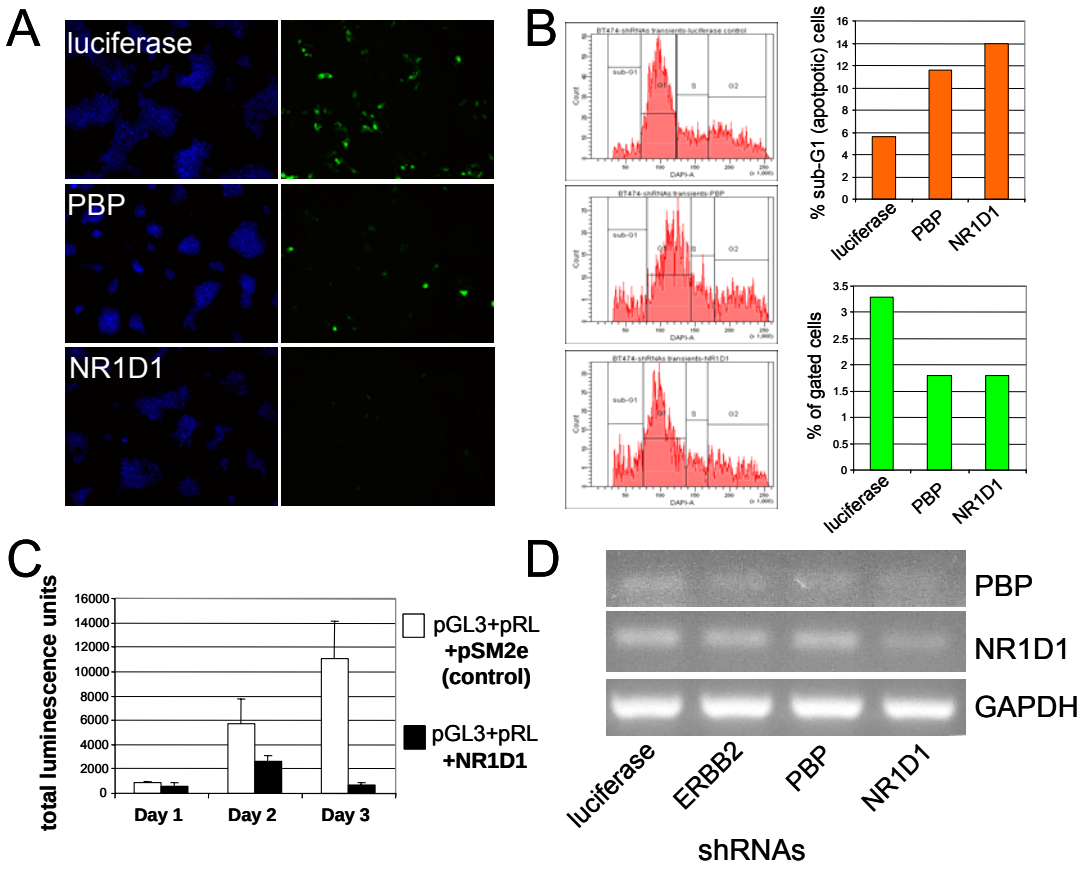

Figure 3. PBP and NR1D1 shRNAs result in decreased viability of BT474 cells (A) BT474s were transfected with the two shRNAs and a luciferase shRNA and co-transfected with MSCV-GFP. After four days cells were fixed with formaldehyde, stained with Hoechst 33243 and imaged under a CCD camera for the ultraviolet (first column) and green (second column) channels. Pictures show a significant decrease of the GFP-positive cells when PBP and NR1D1 hairpins were used, comparing to the luciferase hairpin. **(B)** BT474 cells treated and stained as above were harvested and used for FACS analysis; results were gated for the green-positive cells. PBP and NR1D1 hairpins seem to increase percentage of apoptotic (sub-G1) cells (first column and second column-top panel), and to significantly decrease (second column, bottom panel) the total number of GFP-positive cells. **(C)** BT474 cells were co-transfected with pGL3 plasmid expressing firefly luciferase, pRL plasmid expressing renilla luciferase, and with NR1D1 hairpin or empty (pSM2e) vector. Cells were assayed for total luciferase activity on days 1, 2 and 3; all experiments were performed in triplicate and show a significant decrease of luciferase activity in cells transfected with NR1D1 hairpin, comparing to the empty vector control. **(D)** HEK 293 cells were transfected with luciferase, ERBB2, PBP and NR1D1 hairpins and the message levels of PBP and NR1D1 were assessed by reverse transcriptase PCR (RT-PCR); GAPDH was used as control. PBP and NR1D1 hairpins down-regulate the respected messages; interestingly, PBP mRNA levels may also be decreased by ERBB2 and NR1D1 hairpins, as well.

BT474 cells exhibit an adipocyte-like phenotype

It has been established by a number of studies that PPARγ and NR1D1 are transcriptional factors that promote adipocyte differentiation (Laitinen *et al.*, 2005). It has also been shown that the PPARγ-binding protein, PBP, as well as NR1D1 are overexpressed in a significant proportion of breast cancer tumors (Dressman *et al.*, 2003; Zhu *et al.*, 1999). These two genes are also overexpressed in BT474 cells (Bertucci *et al.*, 2004). For the above reasons, we sought to examine whether these cells exhibit adipocyte-like properties, such as increased lipid storage. For this we used Oil Red O, a fat-soluble diazo dye used for staining neutral triglycerides and lipids. Oil Red O staining of BT474 and other breast cancer cell lines (MDA-MB-361, MCF-7), as well as of primary mammary cells (HMEC) showed that only BT474 cells were stained for lipids (Fig. 3A). In order to further examine the role of the adipogenesis pathway in BT474 cells, we treated the cells with a specific PPARγ antagonist (Fig. 3B). Results showed that cell viability was significantly reduced specifically in BT474, and to a lower extent, in MDA-MB-361 cells (Fig. 3B). These results, combined with the RNAi experiments show that targeting of components of the adipogenesis pathway (PBP, PPARγ, NR1D1) had the most significant impact on the lipid-rich BT474 cell line, implying for a possible relationship of this pathway to breast cancer cell viability.

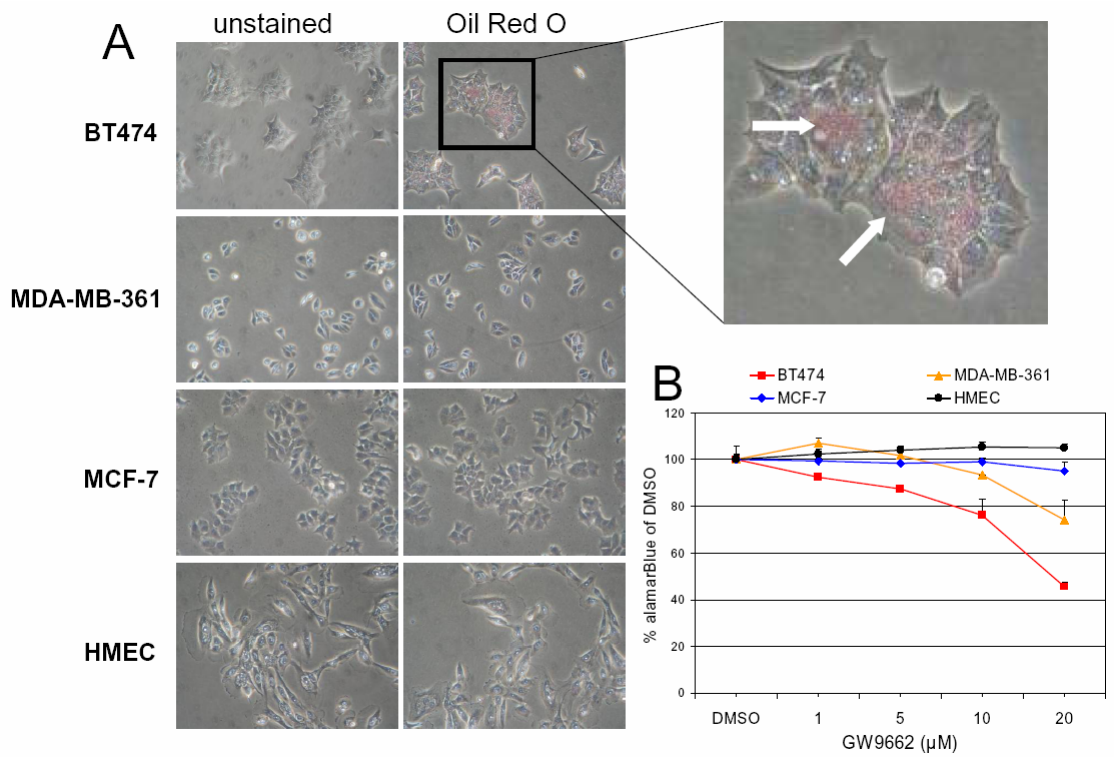

Figure 4. BT474 cells exhibit adipogenic properties (A) Three breast cancer cell lines (BT474, MDA-MB-361 and MCF-7) and human mammary epithelial cells (HMEC) were stained with Oil-Red O for lipid detection; only BT474 cells were stained red, implying the presence of lipid stores. **(B)** The same cell lines were treated with different concentrations of the PPARγ antagonist, GW9662; PPARγ is a major regulator of adipogenesis and is positively regulated by PBP, while it also activates NR1D1. BT474 and, to a lesser extent, MDA-MB-361 cells were significantly affected by the antagonist, as depicted by the use of alamarBlue; interestingly, the level of alamarBlue decrease of BT474 cells at 20 μM of the antagonist, was similar to that achieved by the NR1D1 shRNA (Fig. 1).

PBP and NR1D1 may affect activity of cancer-related circadian clock genes

NR1D1 has been identified as a major regulator of the circadian clock (Yin & Lazar, 2005; Yin *et al.*, 2006; Ueda *et al.*, 2005). NR1D1 down-regulates expression of BMAL1 (Yin & Lazar, 2005), another key circadian component that induces expression of the PER and CRY families of genes, while it also activates NR1D1 expression, by this way establishing positive and negative feedback loops (Yin *et al.*, 2006). Interestingly, a number of recent studies have shown that decreased expression of PER1 and PER2 is associated to tumor aggressiveness (Gery *et al.*, 2006; Fu *et al.*, 2002; Gery *et al.*, 2005). Re-expression of PER1 in several cancer cell lines sensitized cells to DNA damage-induced apoptosis (Gery *et al.*, 2006). In addition, it has been found that the promoters of the PER genes are highly methylated in ERBB2 over-expressing breast tumors (Chen *et al.*, 2005). For these reasons, we wondered whether ERBB2, PBP and NR1D1 knockdown affects expression of circadian genes in BT474 cells. Luciferase reporter assays using PER1 and BMAL1 reporters showed that NR1D1 hairpin significantly induces activity of both genes (Fig. 4), while ERBB2 and PBP hairpins resulted only in a moderate increase of PER1 promoter activity. Therefore, it is seems possible that NR1D1 enhances breast cancer cell aggressiveness via de-regulation of the circadian clock mechanism. It is also possible that restoration of PER1 activity may result in decrease cell viability, as was shown in other cases, yet more evidence is needed towards this direction.

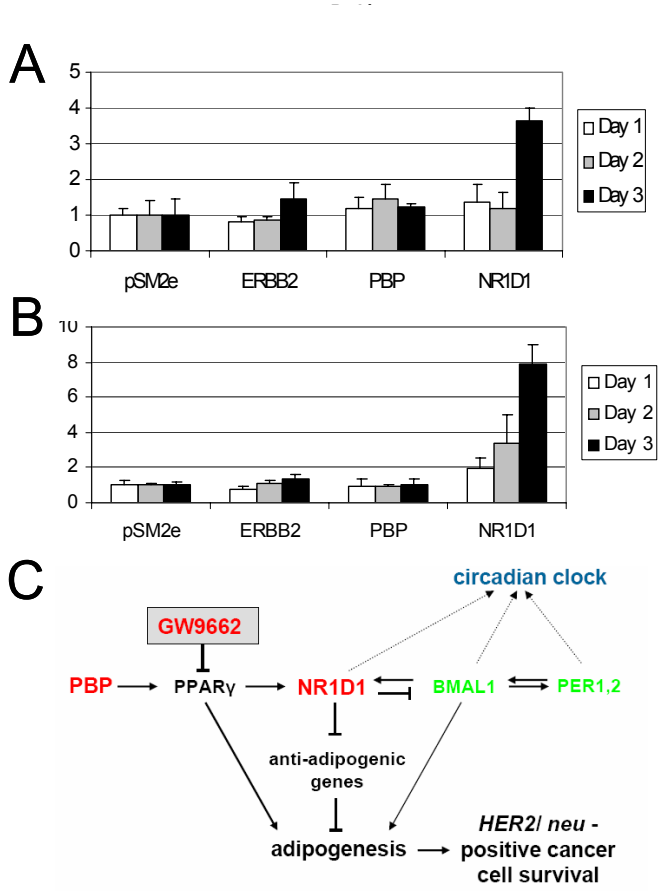

Figure 5. NR1D1 knockdown induced activity of circadian clock genes Per1 (**A**) and Bmal1 (**B**). BT474 cells were transfected with Per1 (**A**) and Bmal1 (**B**) firefly luciferase reporters, pRL plasmid expressing renilla luciferase and with ERBB2, PBP and NR1D1 hairpins or empty vector (pSM2e). Cells were assayed on days 1, 2 and 3 of transfection for firefly luciferase activity; results were normalized for transfection efficiency by measuring renilla activity. Error bars represent standard deviations from a triplicate of experiments. All results are shown as percentage of firefly luciferase of the empty vector control cells. Per1 and Bmal1 activities were significantly increased after NR1D1 knockdown, consistent with both genes being negatively regulated by NR1D1. (**C**) Our current working hypothesis for the importance of PBP and NR1D1 to In addition, Per1 overexpression has been previously shown to negatively affect breast cancer cell viability.

Key Research Accomplishments

Year 1.

- Adopted a new vector and shRNA production methodology
- Created a set of encoded hairpins targeted against genes overexpressed in ErbB-2 positive breast tumors
- Developed a relatively rapid and reliable method for quantification of proliferation changes due to shRNA expression in cells
- Identified approximately 30 genes that negatively impact the growth of Erb-B2 positive breast cancer cells

Year 2.

- **Tested shRNAs that negatively impact the growth of Erb-B2 positive breast cancer cells for effects on normal cells**
- **Tested shRNAs that negatively impact the growth of Erb-B2 positive breast cancer cells for effects on other breast cancer cell lines**
- **Identified approximately 20 shRNAs from the original positives from year 1 that specifically inhibit Erb-B2 positive breast cancer cells target genes. These genes are potential chemotherapeutic targets.**
- **The PBP and NR1D1 genes are related to adipogenesis. Using small molecule inhibitors and reporter experiments, we have developed a working hypothesis that adipogenic genes may be required for breast cancer proliferation. This is likely to be due to its effect on countering cellular hypoxia.**

Year 2 Reportable Outcomes

Manuscripts:

Lastro, M.L., Kourtidis, A. Farley, K. and Conklin, D.S. 2006. Expression of the CD98 light chain xCT reduces the early cell cycle requirement for calcium. *(submitted)*

Evans, S. S. Markham, A. Torres, A. Kourtidis, and D. Conklin 2006. An improved minimum description length learning algorithm for nucleotide sequence analysis. *(submitted)*

Evans, S., A. Kourtidis, T. S. Markham, J. Miller, D. Conklin and A. Torres 2007, microRNA target detection and analysis for genes related to breast cancer using MDLcompress. (*submitted*)

Book chapters:

Kourtidis, A., Eiffert, C., and Conklin, D.S., 2006 RNAi Applications in Target Validation. *Ernst Schering Res Found Workshop*. 2007;(61):1-21

Eifert, C., A. Kourtidis and D. S. Conklin, (2006) RNA interference libraries in dissecting molecular pathways of the human cell, Editor Martin Latterich, *RNAi* (*in press*)

Presentations:

RNAi and Cell Line Engineering, Cell Line Development and Engineering (invited presentation), IBC, San Diego, June 2006

RNAi Applications in Target Validation, Ernst Schering Workshop on Systems Biology, (invited presentation), Berlex / Schering AG, San Francisco, November 2005

RNAi-based mammalian functional genomics, (invited presentation), GE Healthcare Cellular Analysis Symposium, GE Global Research, September 2005

Patents:

Conklin, D.S., A. Kourtidis, and C. Eiffert. Therapeutic targets for breast cancer. (preliminary filing)

Development of cell lines, tissue or serum repositories:

As was proposed, the collection of shRNA constructs targeting overexpressed genes that contribute to breast cancer tumorigenicity will undoubtedly be useful to many investigators and will be made available as soon as testing is complete.

Funding applied for based on work supported by this award:

Submission of an RO1 is planned for June 2007 with preliminary results produced from this project.

Additionally, the following proposals made possible by this award have been submitted:

BC061755 (Evans) 01/01/07-12/31/10
CDMRP-DOD
"Identification and Validation of miRNA Targets Related to Breast Cancer Using an Improved Minimum Description Length (MDL) Sequence Analysis Algorithm",

The major goal of this project is to identify miRNA regulation of genes involved in the genesis of breast cancer.
Role: **Co-investigator**

PDF74806 Postdoctoral Fellowship (Kourtidis) 05/01/06-04/31/09
The Susan G. Komen Breast Cancer Foundation
“Functional Genomic Analysis of Genes Overexpressed in Breast Cancer Using RNAi”

The major goal of this project was to identify the role of HSP70, PBP and NR1D1 genes in breast cancer cell tumorigenicity.
Role: **Mentor**

Postdoctoral Fellowship (Kourtidis) 06/01/06-05/31/09
Life Sciences Research Foundation
“Functional Genomic Analysis of Genes Overexpressed in Breast Cancer Using RNAi”

The major goal of this project was to identify the role of HSP70, PBP and NR1D1 genes in breast cancer cell tumorigenicity.
Role: **Mentor**

Postdoctoral Fellowship (Kourtidis) 06/01/06-05/31/09
Damon Runyon Cancer Research Foundation
“Functional Genomic Analysis of Genes Overexpressed in Breast Cancer Using RNAi”

The major goal of this project was to identify the role of HSP70, PBP and NR1D1 genes in breast cancer cell tumorigenicity.
Role: **Mentor**

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

None in this year.

Year 2 Conclusions

Year 1 of funding resulted in the collation of a large set of constructs targeting genes overexpressed in ErbB-2 positive breast tumors. We have also developed a relatively rapid and reliable method for quantification of proliferation changes due to shRNA expression in cells. Using this we have identified approximately 30 genes that negatively impact the growth of Erb-B2 positive breast cancer cells.

Year 2 of funding resulted in tests of the specificity of the shRNAs' negative impact on the growth of Erb-B2 positive breast cancer cells. Effects of these shRNAs on normal cells and other breast cancer cell lines, identified approximately 20 shRNAs that *specifically* inhibit Erb-B2 positive breast cancer cells target genes. Several of these gene targets are known to be important to a variety of cancers. The PBP and NR1D1 genes are interesting because they give new insight into a pathway that can be exploited as a potential therapeutic target.

"So what?" one might ask.

Our unbiased approach has identified the PBP and NR1D1 genes, which are related to adipogenesis, as genes required by Erb-B2 positive breast cancer for survival. Using small molecule inhibitors and reporter experiments, we have developed a working hypothesis that adipogenic genes may be required for breast cancer proliferation. This is likely to be due to its effect on countering cellular hypoxia.

These genes and the pathway that they operate in are potential chemotherapeutic targets. Our ability to inhibit Erb-B2 positive breast cancer cell proliferation *in vitro* with a small molecule inhibitor of this pathway underscores the potential of this approach.

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